SPECIFICATION

ENVIRONMENTAL STRESS TOLERANT GENE

TECHNICAL FIELD

The present invention relates to DNA encoding proteins having the activity of improving tolerance against environmental stresses such as salt stress and its screening methods, proteins having the activity of improving tolerance against environmental stresses such as salt stress, and the use of the DNA or the proteins such as transgenic plants.

BACKGROUND OF THE INVENTION

Organisms living in the nature are exposed to various environmental stresses such as salt stress, high temperature stress, low temperature stress, freezing stress, or drought stress. Specifically, salt stress is one of the main factors inhibiting the growth of many species of higher plants. Since the improvement of tolerance against salt in higher plants leads to the increase of the farm products, attempts have recently been made vigorously to improve salt tolerance of higher plants by gene introduction.

For example, H. J. Bohnert et al. show that salt tolerance of tobacco plants was improved by introducing the mannitol synthetase derived from coliforms into tobacco plants (Science 259, 22, 508-510, 1993). It has been shown that similar effects of improvement of salt tolerance in higher plants can be obtained by introducing proline synthetase (Plant Physiol. 108, 1387-1394, 1995) or glycine betaine synthetase (Plant J. 12, 133-142, 1997, Plant Mol. Biol. 38, 1011-1019, 1998). However, the recombinant plants obtained by the introduction of the genes encoding the enzymes will not acquire salt tolerance enough to cope with the level of the seawater.

In general, environmental stresses such as salt stress affect on various organic

responses. In other words, in order to produce genetically modified plants capable of growing in an environment at a high level of salt concentration in a stable manner, it is necessary to use gene population encoding proteins having the activity of improving salt tolerance. Main methods used in the past to isolate gene population encoding proteins having the activity of improving environmental stress tolerance have been based on the assumption that "mechanisms resisting to stress express when stress is imposed." More specifically, it detects proteins or mRNA which is specifically expressed when some environmental stress is imposed on a plant, acquires the genes for them following methods of molecular biology, and genetically introducing them to plants weak to such stress, and examines whether the plants become to show tolerance to environmental stress. It is certain that such methods have been used to isolate the genes specifically induced by environmental stress. However, it was rare that plants with a high level of tolerance to environmental stress were made by introducing the genes to plants weak to environment stress. For example, if a gene responsible for salt tolerance expresses with or without stress in a plant growing under a stressful condition such as a high concentration of salt, it is impossible to detect stress tolerance genes in the previous methods. Genome projects have recently been carried out on plants with a high level of stress tolerance. Although their base sequences or sequences of amino acids may be revealed, the present state is that as in other genome projects, there are many proteins whose functions are not identified, and it cannot specify which proteins are responsible for tolerance to environmental stress.

On the other hand, mangroves are woody plants growing in soil containing a high concentration of salt along the coast and near the entry of rivers. Mangrove plants are thought to have acquired special mechanisms for salt tolerance in the process of evolution. Therefore, if we can isolate gene population of mangrove plants responsible for salt tolerance, it is expected that the isolates can be used to apply to improve salt tolerance of higher plants. However, there is no known example of analyzing mechanism of salt tolerance of mangrove plants at genetic level. One of the reasons is that it was quite difficult to extract mRNA of the genes directly involved in

salt tolerance from such woody plants.

Recently, Mimura et al. have established cultured cell lines of Bruguiera sexangula, a kind of mangrove plants (J. Res. 110, 25-29, 1997). The cultured cells are different from other cultured plant cells for their quite specific properties; they can be subjected to suspension culture, and they can grow in a stable manner under the circumstance where the salt concentration is 150mM or more (J. Plant Res. 110, 31-36, 1997). However, it has never been attempted even to detect a group of genes involved in salt tolerance of mangrove plants by constructing cDNA library of mangrove plants with the use of such cultured cells. Moreover, there are few examples of improving salt tolerance of higher plants by introducing the genes derived from other plants with salt tolerance. One representative example is to improve salt tolerance of tobacco plants by introducing inositole methyltransferase genes derived from Masembryanthenum crystallinum, which is a halophyte, into tobacco, and increasing the content of ononitol, a kind of compatible solute, into transformed cells (Plant Physiol. 115, 1221-1219, 1998), and another example is to slightly improve salt tolerance of rice plants by introducing the genes encoding stress inducing proteins (LEA proteins) derived from barley with a relatively high level of salt tolerance, into rice plants (Plant Physiol. 110, 249-257, 1996). As shown above, there is no established technology for isolating effectively a group of genes encoding proteins having the activity of improving tolerance to salt stress, and at the present situation, the environmental stress tolerant genes in many halophytes such as a group of mangrove plants have not been studied well enough.

Further, the functions of improving salt stress tolerance of proteins can be improved by artificially modifying genes encoding the proteins having the activity of improving salt stress tolerance, it becomes possible to produce plants with a higher level of tolerance to salt stress. There was an attempt to stabilize the expression level of choline dehydrogenase in plants by modifying some codons when expressing choline dehydrogenase derived from coliforms in a plant, which leads to stabilization of the level of glycine betaine (a kind of compatible solute, which has a function of improving

salt tolerance in plants), which is a metabolite of choline dehydrogenase (Stress responses of photosynthesis organisms (ed. Satoh K., Murata N.), 115-131, Elsevier Science, Amsterdam). However, this is not the one that changes a sequence of amino acids in proteins. It has never been reported to improve the level of salt stress tolerance of higher plants by introducing proteins whose sequences of amino acids are modified (improved) and whose activity improves salt stress tolerance. Further, it is expected that there are possibilities that the genes or their modified genes involved in salt tolerance have the activity of improving tolerance not only to salt stress but also to all or some of the other kinds of environmental stresses (thermal, freezing, osmotic pressure, drought, and ultraviolet).

DISCLOSURE OF THE INVENTION

The object of the present invention is to provide efficient methods for screening genes having the effects of improving tolerance to environmental stress in various organisms, genes of proteins (proteins having the activity of improving environmental stress tolerance) having the activity of improving environmental stress tolerance obtained from the screening method, proteins having the activity of improving environmental stress tolerance, and transgenic plants or the like whose salt tolerance is improved.

To solve the problem mentioned above, the present inventors focused on mangroves, which are halophytes, obtained a portion of cultured cells of Bruguiera sexangula, which was established as a cultured cell lines, cultured this cell line in the presence of 100mM of NaCl, produced cDNA library based on the mRNA extracted from the cultured cells, and attempted to detect genes involved in salt tolerance in mangroves. Normally, differential screening is widely used to screen genes involved in salt tolerance (see Publication for Japanese Laid-Open Patent Application No. 10-295380 on novel thionine genes induced by salt stress). However, the genes which this screening method isolates are the ones specifically derived under stressful conditions, and it is not

always the case that the expression of the genes in other cells leads to the improvement of stress tolerance of the cells. The present inventors developed methods of using gene expression systems of coliform to detect genes involved in stress tolerance.

When screening genes involved in salt tolerance by using gene expression systems of coliform, a problem is that the defending mechanism of coliform itself strongly works against sodium chloride (NaCl). The coliforms widely used in the field of molecular biology at the presence such as DH5 α, HB101, JM109 have the ability to form colonies even in the 2YT agar medium containing 100mM or more of NaCl. When the screening is carried out with these cell lines, we would obtain not only the clones with their salt tolerance improved by the expression of candidate cDNA derived from the above-mentioned cDNA library but also the clones irrelevant to salt tolerance, since salt tolerance mechanism works strongly in the coliforms themselves, and it is extremely difficult to discern them. For these reasons, the selection of genes relevant to salt tolerance by using gene expression systems of coliform has never been carried out. The present inventors discovered the coliforms where the level of the salt tolerance mechanism becomes low in comparison with that of other coliforms, and succeeded in screening genes relevant to salt tolerance with the use of coliforms for the first time.

It was confirmed that the group of genes (cDNA), derived from halophytes such as mangroves isolated by the present inventors following the above-mentioned method, has functions of improving the salt tolerance in coliform. Since it was possible to improve the level of salt tolerance in the coliforms by expressing the plant genes in coliforms, which are different organisms, a group of such genes are considered to have functions of improving salt tolerance in a wide range of organisms from prokaryotes to eukaryotes. In fact, the present inventors have succeeded in improving the salt tolerance in yeast, plant cells (cultured tobacco cells), and plant organisms (tobacco plants) by introducing a single gene, which is isolated and named mang1 gene, from among the group of genes involved in stress tolerance. It is also confirmed that mang1 has functions of improving the levels of tolerance to environmental stresses such as

thermal, osmotic pressure, freezing other than salt tolerance. Further, we found that it was possible to obtain proteins having stronger activity of improving salt tolerance by introducing random mutants into mangl cDNA, introducing the mutant cDNA into coliforms, and carrying out the process of selecting once, or twice or more under more stringent conditions than the selecting condition before the mutation. The present invention has been accomplished based on the sequence of researches.

The present invention relates to a method for screening DNA encoding proteins having the activity of improving environmental stress tolerance wherein candidate cDNA derived from cDNA library is introduced into host cells, the obtained transformed cells are cultured under the conditions where the host cells cannot substantially grow, the clones grown after culturing are selected, and the candidate cDNA introduced from the selected clones is isolated (claim 1), a method for screening DNA encoding proteins having the activity of improving environmental stress tolerance wherein candidate cDNA derived from cDNA library is introduced into host cells, the obtained transformed cells are cultured under conditions where the host cells cannot substantially grow, the clones grown after the culturing are selected, candidate cDNA is isolated from the selected clones, the isolated candidate cDNA is introduced into the isolated cDNA, the mutant cDNA is introduced into host cells, and the process of selecting is repeated one or more under stringent conditions of selecting mutant cDNA than the selecting condition (claim 2), the method for screening according to any one of claims 1 or 2, wherein the environmental stress is one or more of chemical substance stress, high temperature stress, low temperature stress, freezing stress, drought stress, ozone stress, ultraviolet stress, radiation stress, or osmotic pressure stress (claim 3), the method for screening according to claim 3, wherein the chemical substance stress is salt stress (claim 4), the method for screening according to any one of claims 1 to 4, wherein the host cell is a coliform (claim 5), the method for screening according to claim 5, wherein the coliform is SOLR strain (claim 6), the method for screening according to any one of claims 1 to 6, wherein an environmental condition where host cells cannot substantially grow is 350mM or more of salt concentration (claim 7).

The present invention also relates to DNA encoding proteins having the activity of improving environmental stress tolerance wherein the DNA is obtained according to any one of claims 1 to 7 (claim 8), DNA encoding proteins having the activity of improving environmental stress tolerance according to claim 8, wherein the environmental stress is one or more of stresses selected from chemical substance stress, high temperature stress, low temperature stress, freezing stress, drought stress, ozone stress, ultraviolet stress, radiation stress, or osmotic pressure stress (claim 9), DNA encoding proteins having the activity of improving the environmental stress tolerance according to claim 9, wherein the chemical substance stress is salt stress (claim 10), DNA encoding proteins having the activity of improving the environmental stress tolerance according to any one of claims 8 to 10, wherein the proteins having the activity of improving the environmental stress tolerance are derived from plants (claim 11), DNA encoding proteins having the activity of improving the environmental stress tolerance according to claim 11, wherein the plant is Bruguiera sexangla, Avicennia marina, Sueada japonica, Salsola komarovii, or Mesembryanthemum crystallinum (claim 12), DNA encoding proteins according to any one of the following (a) to (c): (a) a protein comprising the sequence of amino acids shown in Seq. ID No. 2, (b) a protein comprising a sequence of amino acids having 70% or more of homology with the sequence of amino acids shown in Seq. ID No. 2, and having the activity of tolerance at least against salt stress, (c) a protein having a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 2, and having the activity of improving tolerance at least against salt stress (claim 13), DNA comprising part or whole of the sequence of bases shown in Seq. ID No. 1, or its complementary sequence (claim 14), DNA hybridized with the DNA according to claim 14 under stringent conditions, and encoding proteins having the activity of improving tolerance at least against salt stress (claim 15), DNA encoding proteins according to any one of the following (a) or (b): (a) a protein comprising the sequence of amino acids shown in Seq. ID No. 4, (b) a protein comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the

sequence of amino acids shown in Seq. ID No. 4, and having the activity of improving tolerance at least against salt stress (claim 16), DNA comprising part or whole of the sequence of bases shown in Seq. ID No. 3 or its complementary sequence (claim 17), DNA hybridized with the DNA according to claim 17 under stringent conditions, and encoding proteins having the activity of improving tolerance at least against salt stress (claim 18), DNA encoding proteins according to any one of the following (a) or (b): (a) a protein comprising the sequence of amino acids shown in Seq. ID No. 6, (b) a protein comprising the sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 6, and having the activity of improving tolerance at least against salt stress (claim 19), DNA comprising part or whole of the sequence of bases shown in Seq. ID No. 5 or its complementary sequence (claim 20), DNA hybridized with the DNA according to claim 20 under stringent conditions, and encoding proteins comprising the activity of improving tolerance at least against salt stress (claim 21), DNA encoding proteins according to any one of the following (a) or (b): (a) a protein comprising the sequence of amino acids shown in Seq. ID No. 8, (b) a protein comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 8, and having the activity of improving tolerance at least against salt stress (claim 22), DNA comprising part or whole of the sequence of bases shown in Seq. ID No. 7 or its complementary sequence (claim 23), DNA hybridized with the DNA according to Claim 23 under stringent conditions, and encoding proteins having the activity of improving tolerance at least against salt stress (claim 24), DNA encoding proteins according to any one of the following (a) or (b): (a) a protein comprising a sequence of amino acids shown in Seq. ID No. 10, (b) a protein comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 10, and having the activity of improving tolerance at least against salt stress (claim 25), DNA comprising part or whole of the sequence of bases shown in Seq. ID No. 9 or its complementary sequence (claim 26), DNA hybridized with the DNA according to claim

26 under stringent conditions, and encoding proteins having the activity of improving tolerance at least against salt stress (claim 27), DNA encoding proteins according to any one of the following (a) or (b): (a) a protein having the sequence of amino acids shown in Seq. ID No. 12, (b) a protein having a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 12, and having the activity of improving tolerance at least against salt stress (claim 28), DNA comprising part or whole of the sequence of bases shown in Seq. ID No. 11 or its complementary sequence (claim 29), DNA hybridized with the DNA according to claim 29 under stringent conditions, and encoding proteins having the activity of improving tolerance at least against salt stress (claim 30), DNA encoding proteins according to any one of the following (a) or (b): (a) a protein having the sequence of amino acids shown in Seq. ID No. 14, (b) a protein having a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 14, and having the activity of improving tolerance at least against salt stress (claim 31), DNA comprising part or whole of the sequence of bases shown in Seq. ID No. 13 or its complementary sequence (claim 32), DNA hybridized with the DNA according to claim 32 under stringent conditions, and encoding proteins having the activity of improving tolerance at least against salt stress (claim 33), DNA encoding proteins according to any one of the following (a) or (b): (a) a protein comprising the sequence of amino acids shown in Seq. ID No. 16, (b) a protein comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 16, and having the activity of improving tolerance at least against salt stress (claim 34), DNA comprising part or whole of the sequence of bases shown in Seq. ID No. 15 or its complementary sequence (claim 35), DNA hybridized with the DNA according to claim 35 under stringent conditions, and encoding proteins having the activity of improving tolerance at least against salt stress (claim 36), DNA encoding proteins according to any one of the following (a) or (b): (a) a protein comprising the sequence of amino acids shown in Seq. ID No. 18, (b) a protein comprising a sequence of amino acids wherein

one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 18, and having the activity of improving tolerance at least against salt stress (claim 37), DNA having part or whole of the sequence of bases shown in Seq. ID No. 17 or its complementary sequence (claim 38), DNA hybridized with the DNA according to Claim 38 under stringent conditions, and encoding proteins having the activity of improving tolerance at least against salt stress (claim 39), DNA encoding proteins according to any one of the following (a) or (b): (a) a protein comprising the sequence of amino acids shown in Seq. ID No. 20, (b) a protein comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 20, and having the activity of improving tolerance at least against salt stress (claim 40), DNA comprising part or whole of the sequence of bases shown in Seq. ID No. 19 or its complementary sequence (claim 41), DNA hybridized with the DNA according to claim 41 under stringent conditions, and encoding proteins having the activity of improving tolerance at least against salt stress (claim 42), DNA encoding proteins according to any one of the following (a) or (b): (a) a protein comprising the sequence of amino acids shown in Seq. ID No. 22, (b) a proteins comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 22, and having the activity of improving tolerance at least against salt stress (claim 43), DNA comprising part or whole of the sequence of bases shown in Seq. ID No. 21 or its complementary sequence (claim 44), DNA hybridized with the DNA under stringent conditions according to claim 44, and encoding proteins having the activity of improving tolerance at least against salt stress (claim 45), DNA encoding proteins according to any one of the following (a) or (b): (a) a protein comprising the sequence of amino acids shown in Seq. ID No. 24, (b) a protein comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 24, and having activity of improving tolerance at least against salt stress (claim 46), DNA comprising part or whole of the sequence of bases shown in Seq. ID No. 23 or its complementary sequence (claim 47),

DNA hybridized with the DNA according to Claim 47 under stringent conditions, and encoding proteins having the activity of improving tolerance at least against salt stress (claim 48), DNA encoding proteins according to any one of the following (a) or (b): (a) a protein comprising the sequence of amino acids shown in Seq. ID No. 26, (b) a protein comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 26, and having the activity of improving tolerance at least against salt stress (claim 49), DNA comprising part or whole of the sequence of bases shown in Seq. ID No. 25 or its complementary sequence (claim 50), DNA hybridized with the DNA according to Claim 50 under stringent conditions, and encoding proteins having the activity of improving tolerance at least against salt stress (claim 51), DNA encoding proteins according to any one of the following (a) or (b): (a) a protein comprising the sequence of amino acids shown in Seq. ID No. 28, (b) a protein comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 28, and having the activity of improving tolerance at least against salt stress (claim 52), DNA comprising part or whole of the sequence of bases shown in Seq. ID No. 27 or its complementary sequence (claim 53), DNA hybridized with the DNA according to claim 53 under stringent conditions, and encoding proteins having the activity of improving tolerance at least against salt stress (claim 54), DNA encoding proteins according to any one of the following (a) or (b): (a) a protein comprising the sequence of amino acids shown in Seq. ID No. 30, (b) a protein comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in. ID No. 30, and having the activity of improving tolerance at least against salt stress (claim 55), DNA comprising part or whole of the sequence of bases shown in Seq. ID No. 29 or its complementary sequence (claim 56), DNA hybridized with the DNA according to Claim 56 under stringent conditions, and encoding proteins having the activity of improving tolerance at least against salt stress (claim 57), DNA encoding proteins according to any one of the following (a) or (b): (a) a protein comprising the sequence

of amino acids shown in Seq. ID No. 32, (b) a protein comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 32, and having the activity of improving tolerance at least against salt stress (claim 58), DNA comprising part or whole of the sequence of bases shown in Seq. ID No. 31 or its complementary sequence (claim 59), DNA hybridized with the DNA according to claim 59 under stringent conditions, and encoding proteins having the activity of improving tolerance at least against salt stress (claim 60), DNA encoding proteins according to any one of the following (a) or (b): (a) a protein comprising the sequence of amino acids shown in Seq. ID No. 34, (b) a protein comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 34, and having the activity of improving tolerance at least against salt stress (claim 61), DNA comprising part or whole of the sequence of bases shown in Seq. ID No. 33 or its complementary sequence (claim 62), DNA hybridized with the DNA according to claim 62 under stringent conditions, and encoding proteins having the activity of improving tolerance at least against salt stress (claim 63), DNA encoding proteins according to any one of the following (a) or (b): (a) a protein comprising the sequence of amino acids shown in Seq. ID No. 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, or 64, (b) a protein comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, or 64, and having the activity of improving tolerance at least against salt stress (claim 64), DNA comprising part or whole of the sequence of bases shown in Seq. ID No. 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, or 63, or its complementary sequence (claim 65), and DNA hybridized with the DNA according to claim 65 under stringent conditions, and encoding proteins having the activity of improving tolerance at least against salt stress (claim 66).

The invention also relates to a method for improving environmental stress tolerance, wherein the DNA according to any one of claims 8 to 66 is used (claim 67),

the method for improving the environmental stress tolerance according to claim 67, wherein the environmental stress is one or more of chemical substance stress, high temperature stress, low temperature stress, freezing stress, drought stress, ozone stress, ultraviolet stress, radiation stress, and/or osmotic pressure stress (claim 68), and the method for improving environmental stress tolerance according to claim 68, wherein the chemical substance stress is salt stress (claim 69).

The invention also relates to a protein comprising of the sequence of amino acids shown in Seq. ID No. 2 (claim 70), a protein having 70% or more of homology with the sequence of amino acids shown in Seq. ID No. 2, and having the activity of improving tolerance at least against salt stress (claim 71), a protein comprising a sequence of amino acids wherein one or more amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 2, and having the activity of improving tolerance at least against salt stress (claim 72), a protein comprising the sequence of amino acids shown in Seq. ID No. 4 (claim 73), a protein comprising a sequence of amino acids wherein one or more amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 4, and having the activity of improving tolerance at least against salt stress (claim 74), a protein comprising the sequence of amino acids shown in Seq. ID No. 6 (claim 75), a protein comprising a sequence of amino acids wherein one or more amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 6, and having the activity of improving tolerance at least against salt stress (claim 76), a protein comprising the sequence of amino acids shown in Seq. ID No. 8 (claim 77), a protein comprising a sequence of amino acids wherein one or more amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 8, and having the activity of improving tolerance at least against salt stress (claim 78), a protein comprising the sequence of amino acids shown in Seq. ID No. 10 (claim 79), a protein comprising a sequence of amino acids wherein one or more amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 10, and having the activity of improving tolerance at least against salt stress (claim 80), a protein comprising the sequence of

amino acids shown in Seq. ID No. 12 (claim 81), a protein comprising a sequence of amino acids wherein one or more amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 12, and having the activity of improving tolerance at least against salt stress (claim 82), a protein comprising the sequence of amino acids shown in Seq. ID No. 14 (claim 83), a protein comprising a sequence of amino acids wherein one or more amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 14, and having the activity of improving tolerance at least against salt stress (claim 84), a protein comprising the sequence of amino acids shown in Seq. ID No. 16 (claim 85), a protein comprising a sequence of amino acids wherein one or more amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 16, and having the activity of improving tolerance at least against salt stress (claim 86), a protein comprising the sequence of amino acids shown in Seq. ID No. 18 (claim 87), a protein comprising a sequence of amino acids wherein one or more amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 18, and having the activity of improving tolerance at least against salt stress (claim 88), a protein comprising the sequence of amino acids shown in Seq. ID No. 20 (claim 89), a protein comprising a sequence of amino acids wherein one or more amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 20, and having the activity of improving tolerance at least against salt stress (claim 90), a protein comprising the sequence of amino acids shown in Seq. ID No. 22 (claim 91), a protein comprising a sequence of amino acids wherein one or more amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 22, and having the activity of improving tolerance at least against salt stress (claim 92), a protein comprising the sequence of amino acids shown in Seq. ID No. 24 (claim 93), a protein comprising a sequence of amino acids wherein one or more amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 24, and having the activity of improving tolerance at least against salt stress (claim 94), a protein comprising the sequence of amino acids shown in Seq. ID No. 26 (claim 95), a protein comprising a sequence of

amino acids wherein one or more amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 26, and having the activity of improving tolerance at least against salt stress (claim 96), a protein comprising the sequence of amino acids shown in Seq. ID No. 28 (claim 97), a protein comprising a sequence of amino acids wherein one or more amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 28, and having the activity of improving tolerance at least against salt stress (claim 98), a protein comprising the sequence of amino acids shown in Seq. ID No. 30 (claim 99), a protein comprising a sequence of amino acids wherein one or more amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 30, and having the activity of improving tolerance at least against salt stress (claim 100), a protein comprising the sequence of amino acids shown in Seq. ID No. 32 (claim 101), a protein comprising a sequence of amino acids wherein one or more amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 32, and having the activity of improving tolerance at least against salt stress (claim 102), a protein comprising the sequence of amino acids shown in Seq. ID No. 34 (claim 103), a protein comprising a sequence of amino acids wherein one or more amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 34, and having the activity of improving tolerance at least against salt stress (claim 104), a protein comprising the sequence of amino acids shown in Seq. ID No. 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, or 64 (claim 105), and a protein comprising a sequence of amino acids wherein one or more amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, or 64, and having the activity of improving tolerance at least against salt stress (claim 106).

The present invention also relates to an antibody specifically bound to the protein according to any one of claims 70 to 72 (claim 107), an antibody specifically bound to the protein according to any one of claims 73 to 104 (claim 108), an antibody specifically bound to the protein according to any one of claims 105 or 106 (claim 109), and the antibody according to any one of claims 107 to 109, wherein the antibody is a

monoclonal antibody (claim 110).

The present invention also relates to a vector comprising the DNA encoding proteins having the activity of improving tolerance against environmental stresses according to any one of claims 8 to 12 (claim 111), a vector comprising the DNA according to any one of claims 13 to 15 (claim 112), a vector comprising the DNA according to any one of claims 16 to 63 (claim 113), and a vector comprising the DNA according to any one of claims 64 to 66 (claim 114).

The present invention also relates to a transformed cell obtained by introducing the vector according to any one of claims 111 to 114 (claim 115), a transformed cell according to claim 115, wherein the host cell is a plant cell (claim 116), and a method for producing proteins having the activity of improving environmental stress tolerance, wherein the transformed cells according to either of claims 115 or 116 is cultured, and recombinant proteins are collected from the transformed cells or the supernatant of the cultured liquid (claim 117).

The present invention also relates to a transgenic plant obtained by introducing the DNA according to any one of claims 8 to 12 encoding proteins having the activity of improving environmental stress tolerance, and by dividing, proliferating and redifferentiating the plant cell (claim 118), a transgenic plant obtained by introducing the DNA according to any one of claims 13 to 15 encoding proteins having the activity of improving environmental stress tolerance, and by dividing, proliferating and redifferentiating the plant cell (claim 119), a transgenic plant obtained by introducing the DNA according to any one of claims 16 to 63 encoding proteins having the activity of improving environmental stress tolerance, and by dividing, proliferating and redifferentiating the plant cell (claim 120), a transgenic plant obtained by introducing the DNA according to any one of claims 64 to 66 encoding proteins having the activity of improving environmental stress tolerance, and by dividing, proliferating and redifferentiating the plant cell (claim 121), a transgenic plant obtained by introducing the vector according to any one of claims 111 to 114, and by dividing, proliferating and redifferentiating the plant cell (claim 122), the transgenic plant according to any one of

claims 118 to 122, wherein the environmental stress is one or more of chemical substance stress, high temperature stress, low temperature stress, frenzying stress, drought stress, ozone stress, ultraviolet stress, radiation stress, and/or osmotic pressure stress (claim 123), the transgenic plant according to claim 123, wherein the chemical substance stress is salt stress (claim 124), and a material for breeding derived from the transgenic plant according to any one of claims 118 to 122 (claim 125).

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the result of detecting salt tolerance of coliforms (SOLR) wherein mang1 is introduced. Colony forming was used as an index for detection. As a control, a vector (pBluescript SK) was used alone. Measurement was carried out with two levels of salt (NaCl) concentration.

Fig. 2 shows the result of detecting salt tolerance of the coliforms wherein various parts of a sequence of mang1 were introduced. Colony forming was used as an index for detection. As a control, a vector (pBluescript SK) was used alone. Measurement was carried out with two levels of salt (NaCl) concentration. The numbers in the parentheses indicate the numbers of amino acids, and "*" indicates cDNA of mang1 containing coding and non-coding regions.

Fig. 3 shows the result of measuring the time-course growth of the yeast introduced with mang1 under the condition of high concentration of salt. Cell concentration was used as an index for detection. As a control, a vector (pYES2) was used alone. Measurement was carried out with two levels of salt (NaCl) concentrations.

Fig. 4 shows the result of measuring the growth of the tobacco-cultured cells wherein mang1 was introduced under the condition of high concentration of salt. Wet weight was used as an index for detection. As a control, a vector (GUS) was used alone. Measurement was carried out with three concentrations of salt (NaCl).

Fig. 5 shows the result of measuring the growth of the tobacco plant organisms wherein mang1 was introduced under the condition of high concentration of salt (150mM of

NaCl). A and C indicate the tobaccos wherein a vector (GUS) alone was introduced, and B and D indicate the tobaccos wherein mangl is introduced.

Fig. 6 shows the result of examining thermal stress tolerance of coliforms (SOLR) wherein mangl was introduced. The thermal stress tolerance was evaluated with the growing curve in the culture at 40 °C as an index. As a control, the SOLR introduced with a vector (pBluesript SK) alone was used.

Fig. 7 shows the result of examining the osmotic pressure tolerance of the coliforms (SOLR) wherein mangl was introduced. The osmotic pressure tolerance was evaluated with the growth on 2YT agar medium containing 800mM of sorbitol as an index. As a control, the SOLR introduced with a vector (pBluescript SK) alone was used.

Fig. 8 shows the result of examining the freezing stress tolerance of coliforms (SOLR) wherein mangl was introduced. The freezing stress tolerance was evaluated with the growth of cell bodies treated with freezing and melting method on 2YT agar medium. As a control, a SOLR wherein only vectors (pBluescript SK) were introduced was used.

Fig. 9 shows a region considered as minimal functional region of mangl (mangl core), and the sequences of bases and amino acids of clones wherein mutants were introduced. The white letters in the sequences of bases and amino acids shows the mutated positions.

Fig. 10 shows the regions considered as minimal functional region of mangl, and the result of the growth of the clones, the regions of which are introduced with mutation, on the agar mediums containing 85mM, 350mM, and 500mM of NaCl. As a control, SOLR introduced with a vector (pBluescript) was used alone.

BEST MODE TO CARRY OUT THE INVENTION

Any method for screening DNA encoding proteins having activity of improving environmental stress tolerance in the present invention can be employed as long as it introduces candidate cDNA derived from cDNA library into host cells, cultures obtained transformed cells under the conditions where host cells are substantially unable

to grow, selects clones growing after cultivation, and isolates candidate cDNA introduced from the selected clones. This screening method can be used to improve functions of proteins having activity of improving environmental stress tolerance obtained by this method. In other words, a method for screening DNA encoding proteins having activity of improving other types of environmental stress tolerance in the present invention can be exemplified by a screening method wherein candidate cDNA derived from cDNA library was introduced into host cells, the obtained transformed cells are cultured under the environment where host cells cannot substantially grow, the matured clones were selected after the cultivation, the selected clones candidate cDNA was isolated from the selected clones wherein the cDNA was introduced, random mutants were introduced into isolated candidate cDNA, the mutant cDNA was introduced into host cells, and the process of selection under more stringent conditions than the condition for selecting cDNA before mutation was repeated once or more.

As a method for introducing random mutants into optionally selected gene fragments in a method of improving function of proteins having activity of improving environmental stress tolerance by repeating the process of selection under more stringent conditions than the ones where the genes are screened from the products wherein random mutants are introduced into gene fragments obtained as a result of the first screening, PCR is a method generally used, and the method to lower the fidelity by adding manganese to PCR reactive solution (A Journal of Methods in Cell and Molecular Biology 1, 11-15, 1989, Yeast 8, 79-82, 1992) is the easiest one. DNA shuffling (Proc Natl Acad Sci USA 91, 10747-10751, 1994) can also be used as another method for random mutation.

Environmental stress mentioned above can be any kind of stress based on environmental factors, including chemical substance stress, high-temperature stress, low-temperature stress, freezing stress, drought stress, ozone stress, ultraviolet stress, radiation stress, osmotic pressure stress, and such environmental stresses can be based on either one single factor or a plurality of stresses. Further, a chemical substance

stress can be any stress caused by chemical substance, and is exemplified by salt stress or toxic substance stress.

There are no restrictions on the derivations of the cDNA such as plants, animals, microorganisms, and others as long as it includes cDNA of the gene involved in environmental stress, as the above mentioned cDNA library. For example, when screening DNA encoding proteins having activity of improving salt stress tolerance, cDNA library prepared from organic species such as halophilous plants, including mangroves (Sonneatia, Bruguiera, Kandelia, Rhizophora, Lumnitzera, Salsola komarovii, Nypa), Mesembryanthemum, Sueada japonica, Aster tripolium, Salicornia, Suaeda, and Atriplex subcordata Kitag can be used. A method of preparing cDNA library can be any one well known to a person having ordinary skill in the art. For example, as disclosed in the examples, extraction of total mRNA from cells can be prepared following the method in Ostrem et al. (Plant Physiol. 84, 1270-1275, 1987), and poly(A) +RNA can be purified from mRNA prepared with Oligotex-dT30<super> (Daiichi Kagakusha). The cDNA library can be constructed by using ZAP-cDNA/Gigapack Cloning Kit (Stratagen) based on the purified poly(A) +RNA.

Although a host cell wherein candidate cDNA was introduced as a target of screening derived from the cDNA library, can be a cell in microorganism such as bacteria or yeast, or a cell in animals or plants, it is preferable to use animal cells whose knowledge on the host-vector system has been established, such as coliforms bacillus, Bacillus subtilis, Saccharomyces cerevisiae, BHK cells and the like. Among them, it is desirable to use coliforms bacillus because knowledge about it is abundant, it grows fast, and it is easy to handle. As a host cell, it is preferred to use a cell which cannot substantially grow under the condition where a transformed cell wherein candidate cDNA derived from cDNA library can grow, such as a salt sensitive cell or a thermal salt sensitive cell. Such cells can be prepared by screening or mutating wild type strains.

Next, transformation of cells can be performed by introducing candidate cDNA derived from cDNA library into the host cell, and such a method of introduction can be

any well-known method of gene introduction such as transformation method or electroporation. The transformed cells wherein candidate cDNA is introduced are cultured under environment where the host cells cannot substantially grow, including under the condition of high concentration of salt, the condition of high temperature, the condition of drought. The matured clones can be selected by well known methods after cultivation, and candidate cDNA introduced from the selected clones can be isolated by the method.

The screening method in the present invention will be described in more details with examples where the environmental stress is salt stress and the host cell is coliform. As a coliform used as a host cell, it is preferable to use a coliform whose function of salt tolerance is degraded, and more preferably a coliform with a minimal concentration of salt inhibiting their growth. For example, it is preferred to use coliforms whose ability of forming a colony in a culture medium containing more than 750mM of NaCl, preferably more than 500 mM of NaCl, and most desirably more than 350mM of NaCl, in view of improving the efficiency of screening genes relevant to salt tolerance. A coliform sensitive to NaCl can be exemplified by SOLR strain (commercialized by TOYOBO, Stratagene, Riken gene bank, and others), a kind of coliform which the present inventors found, that it cannot grow on the agar medium containing a low level of salt (more than 350mM of NaCl). When using this SOLR strain, the SOLR strain contained in said ZAP-cDNA/Gigapack Cloning Kit (Stratagene) and in vivo excision system by ExAssist helper phage can be used for the introduction of candidate cDNA. All of these operations can easily be performed following the user's manual for the kit.

The SOLR strain obtained in this ways wherein the candidate cDNA is introduced is cultured in a medium containing about 400mM of NaCl and the matured cells are selected to select clones transformed by the DNA encoding proteins having salt tolerance. For example, such selection of clones may be carried out by culturing on agar medium for 8 to 20 hours at 37°C and select colonies formed on the agar. For example, as a method for isolating cDNA from the selected clones, a method disclosed in a reference (Current Protocols in Molecular Biology (Greene Publishing Associates

and Wiley-Interscience, 1987) can be performed by extracting plasmid DNA.

The screening to obtain the targeted transformed coliforms can be carried out repeatedly for several times. For example, the coliforms wherein cDNA library was introduced are cultured in a medium containing the minimal concentration of salt inhibiting their growth, and select the clones which can grow under this condition (first screening). Next, the cDNA are isolated from the selected clones , and are reintroduced into coliforms. The coliforms are cultured in a medium containing a higher concentration of salt than the minimal concentration of salt inhibiting their growth, and the clones which can grow under this condition are selected (second screening). By repeating such process of screening, the efficiency of isolating the genes relevant to salt tolerance can be improved. Further, as mentioned above, the second screening can be carried out by using mutant cDNA wherein random mutants are introduced into isolated cDNA.

There are no particular restrictions on the DNA encoding proteins having activities of improving the environmental stress tolerance in the present invention as long as the DNA can be obtained by the method for screening, and it can be exemplified by the DNA encoding proteins having activities of improving tolerance against one or more of chemical stress such as salt stress, thermal stress, drought stress, ozone stress, ultraviolet stress, radiation stress, osmotic pressure stress. Specifically, DNA encoding proteins having activities of improving salt stress tolerance can be exemplified as DNA derived from plants, preferably DNA derived from halophytes such as Brugfuiera sexangula. An example of mangrove derived DNA encoding proteins having activities of improving salt stress tolerance can be exemplified as DNA having a sequence of bases shown in Seq. ID No. 1, 3, 5, 7, 9, 11, or 13 in the list of sequences. An example of Mesembryanthemum crystallinum derived DNA encoding proteins having activity of improving salt stress tolerance can be exemplified as DNA having a sequence of bases shown in Seq. ID No. 15, 17, 19, 35, 63 in the list of sequences. An example of Sueada japonica derived DNA encoding proteins having activity of improving salt stress tolerance can be exemplified as DNA having a sequence of bases shown in Seq. ID No.

21, 37, 39, 51, 53, 57 in the list of sequences. An example of Salsola komarovii derived DNA encoding proteins having activity of improving salt stress tolerance can be exemplified as DNA having a sequence of bases shown in Seq. ID No. 23, 25, 41, 47, 49, 59, 61 in the list of sequences. An example of Avicennia marina (a kind of mangrove) derived DNA encoding proteins having activities of improving salt stress tolerance can be exemplified as DNA having a sequence of bases shown in Seq. ID No. 27, 29, 31, 33, 43, 43, 55 in the list of sequences.

DNA in the present invention can be exemplified as DNA having part or whole of a sequence of bases shown in the Seq. ID No. in the list of sequences or their complementary sequences, DNA which can hybridize with the DNA under stringent conditions and encodes proteins having activities of improving tolerance at least against salt stress, DNA which encodes proteins having a sequence of amino acids shown in Seq. ID No. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, or 64 in the list of sequence, DNA which comprises a sequence of amino acids having 70% or more of homology with the sequence of amino acids shown in Seq. ID No. 2 in the list of sequence and encodes proteins having activity of improving tolerance at least against salt stress, or DNA which comprises a sequence of amino acids wherein one or more of the amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, or 64 in the list of sequence, and encodes proteins having activities of improving tolerance at least against salt stress. It has not been reported that tolerance to salt or the like is improved by introducing the DNA into various organisms, and it is found by the present inventors for the first time.

Proteins in the present invention can be exemplified as proteins having a sequence of amino acids shown in Seq. ID No. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, or, 64 in the list of sequence, proteins which have a sequence of amino acids having 70% or more of homology with the sequence of amino acids shown in Seq. ID No. 2 in the list of sequence and have

activity of improving tolerance at least against salt stress, or proteins which have a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, or 64 in the list of sequence and have activity of improving tolerance at least against salt stress.

The sequences of amino acids shown in 8, 14, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, or 64 in the Seq. ID No. are thought not to be the ones encoding the full length of proteins. However, since there is activity of improving salt stress tolerance in itself, they are considered to be a functional region in each of the proteins of full length. As mentioned above, the present invention involves the full length of proteins involving these functional regions and DNA encoding the full length of proteins. As an example of methods for isolating the full cDNA based on partial length of cDNA, it is appropriate to use the kits such as Marathon cDNA Amplification Kit (Clontech), 3'-Full RACE Core Set (TAKARA), 5'-Full RACE Core Set (TAKARA) and follow their user's manuals.

As shown above, the present invention involves DNA encoding proteins functionally equivalent to the proteins having a sequence of amino acids shown in Seq. ID No. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, or 64. It is well known that although a few amino acids are substituted, deleted, or added, proteins having biogenic activity can maintain the biogenic activity. Various methods for mutating amino acids in proteins are well known, and some kits are already commercialized as well. For example, it is easy to mutate amino acids in proteins by synthesizing primers wherein mutants are introduced and with the use of QuikChange Site-Directed Mutagenesis Kit (Stratagene).

Proteins having amino acids wherein one or more of amino acids are substituted, deleted, added and/or inserted in the list of a sequence of amino acids shown in Seq. ID No. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, or 64 are all involved in the scope of the present invention as

long as they have activity of improving environmental stress tolerance at least against salt stress tolerance in various organic cells (for example, plant cells, coliforms, yeast). DNA encoding proteins functionally equivalent to proteins having amino acids shown in Seq. ID No. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, or 64 can be prepared by hybridization technology or molecular amplification technology (Molecular Cloning, a Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989). For example, it is possible to obtain DNA or the like encoding proteins having activity of improving environmental stress tolerance including at least salt stress tolerance as an object by the hybridization of cDNA library derived from various organisms under stringent conditions where the probes are part or whole of a sequence of bases shown in Seq. ID No. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, or 63 encoding a sequence of amino acids shown in Seq. ID No. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, or 64 and the isolation of DNA which hybridizes with the probes.

As a condition for the hybridization to obtain the DNA, hybridization can be carried out at 42°C and treatment of washing at 42° C with the use of washing buffer containing 1×SSC and 0.1 % of SDS. More preferably, it can be hybridized at 65° C and treatment of washing at 65° C with the use of washing buffer containing 0.1×SSC and 0.1 % of SDS. As for factors effecting the stringency of hybridization, there are various other factors other than the thermal ones mentioned above, and a person having ordinary skill in the art is capable of carrying out the same level of stringency as illustrated in the stringency of hybridization by combining various factors in an appropriate way.

It is possible to obtain the DNA or the like encoding proteins having activity of improving environmental stress tolerance including tolerance at least against salt tolerance by polymerase chain reaction of various organism-derived DNA (or RNA) as templates by using oligonucleotides prepared based on a sequence of bases shown in

Seq. ID No. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, or 63 as primers. The present invention involves DNA which can be isolated by using hybridization technology or gene amplification technology, as long as they have activities of improving environmental stress tolerance including at least salt stress tolerance in various organic cells (such as plant cells, coliforms, or yeast).

It is thought that proteins functionally equivalent to proteins having a sequence of amino acids shown in Seq. ID No. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, or 64 have high homology with the sequence of amino acids shown in the sequence numbers. The high level of homology stands for 70 % or more, preferably 80% or more, or more preferable 90% or more (for example, 95% or more) of homology in sequences. The present invention involves DNA whose sequence of amino acids has a high level of homology with the sequence of amino acids shown in the above sequence numbers, and which encodes proteins having activity of improving tolerance at least to salt stress in various organic cells (such as plant cells, coliforms, or yeast). For example, the sequence of amino acids in Seq. ID No. 2 have activity of improving salt stress tolerance in the region comprising amino acids from 1 to 86, and therefore all of the gene DNA encoding a sequence of amino acids comprising this region, for example, are all included in the scope of the present invention. The homology in sequences can be determined, for example, by using the multi-alignment function of GENETYX-MAC (Software Development Corporation), a genetic information processing software.

There are no particular restrictions on a method for improving environmental stress tolerance in the present invention, as long as it uses DNA encoding proteins having activity of improving tolerance against one or more environmental stresses selected from chemical substance stress such as salt stress, high temperature stress, low temperature stress, freezing stress, drought stress, ozone stress, ultraviolet stress, radiation stress, osmotic pressure stress. By the method for improving environmental stress tolerance, environmental stress tolerance can be improved in tissues, organs, and

cells of plants and animals, and microorganisms such as bacteria, yeast, and fungi.

As an antibody specifically binding to proteins having activity of improving tolerance against one or more environmental stresses such as chemical substance stress such as salt stress, thermal stress, drought stress, ozone stress, ultraviolet stress, radiation stress, osmotic pressure stress, any antibody can be used as long as the antibody can specifically bind to proteins in the present invention. Such antibodies can be exemplified as immune specific antibodies such as monoclonal antibiotics, polyclonal antibiotics, chimera antibiotics, single chain antibody, or human cell line antibody or the like. As for the antibodies used for preparation, these antibodies can be produced by using the following proteins having a sequence of amino acids shown in Seq. ID No. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, or 64, proteins having a sequence of 70% or more of homology with a sequence of amino acids shown in Seq. ID No. 2 and having activity of improving tolerance at least against salt stress, and proteins having a sequence of amino acids wherein one or more the amino acids are deleted, substituted, or added in a sequence of amino acids shown in Seq. ID No. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62 or 64 and having activities of tolerance at least against salt stress. Such antibodies are useful in elucidating the molecular structure of proteins having activity of improving environmental stress tolerance and others.

Following the conventional protocols, antibodies for proteins having activity of improving the environmental stress tolerance are produced by administering proteins having activity of improving the environmental stress tolerance in animals (preferably, except for human), or fragments, their analogues or cells involving epitope. As a technology for preparing monoclonal antibodies, any technology can be used such as hybridoma technology (Nature 256, 495-497, 1975), trioma technology, human B cell hybridoma technology (Immunology Today 4, 72, 1983) or EBV-hybridoma technology (MONOCLONAL ANTIBODIES AND CANCER THERAPY, 77-96, Alan R. Liss. Inc., 1985), which produce antibodies from the culture of successive cell strains.

The method for preparing single chain antibodies (US4946778) can apply to produce single chain antibodies for proteins having activity of improving the environmental stress tolerance in the present invention. Further, transgenic plants or transgenic animals and the like can be used to express human cell line antibodies, clones expressing proteins having activity of improving the environmental stress tolerance by using the antibodies can be isolated or identified, or the polypeptides can be purified by using affinity chromatography.

There are no special restrictions on vectors used in the present invention, as long as the vectors comprising DNA encoding proteins having activity of improving tolerance against the environmental stresses such as chemical substance stress such as salt stress, thermal stress, low temperature stress, freezing stress, drought stress, ozone stress, ultraviolet stress, radiation stress, and osmotic pressure stress. There are also no restrictions on transformed cells used in the present invention, as long as the transformed cells are obtained by introducing the vectors into host cells such as plant cells or the like. As a method for producing proteins having activity of improving environmental stress tolerance in the present invention, it can be any method as long as the transformed cells are cultured, and recombinant proteins are collected from the transformed cells or the supernatant of the cultured liquid. Further, any transgenic plant can be used in the present invention as long as it introduces DNA encoding proteins having activity of improving the environmental stress tolerance or the vectors into plant cells, and are obtained by division, proliferation, and redifferentiation of the plant cells. The following explains vectors, transformed cells, methods for producing proteins having activity of improving environmental stress tolerance, and transgenic plant in the present invention.

As mentioned above, DNA in the present invention can be used in preparing recombinant proteins. To prepare recombinant proteins, the DNA in the present invention are inserted into appropriate expression vectors, the vectors are introduced into appropriate host cells, the DNA are expressed, and then the expressed proteins are collected from the transformed cells or the supernate of the cultured liquid. The host-

vector system used in expressing recombinant proteins can be exemplified as IMPACT-CN System (host cell: E. coli strain ER2566, vector:pTYB1, pYB2, pYB11, pYB12 (BioLabs), or pET Expression System (host cell: Epicurian Coli BL 21, vector: pET3 series (Novagen)). As for a method for introducing vectors into host cells, well known methods can be used such as electroporation method or heat shock method (IDENSHI LIBRARY NO SAKUSEIHOO (Methods of producing gene library), Yoodo-sha, 1994, SHOKUBUTU SAIBOO KOOGAKU NYUMON (Introduction to plant cell engineering), Japan Scientific Societies Press, 1998). The culture for expressing recombinant proteins can be carried out by conventional methods and under conventional conditions. Expressed proteins can be purified by Chitin Beads (BioLabs) when IMPACT-CN System is used, and His Bind Resin (Novagen) when pET Expression System is used.

DNA in the present invention can be used to produce transgenic organisms whose tolerance at least against salt stress is improved. There are no restrictions on the species of organisms in producing transgenic organisms by using DNA in the present invention. However, in the case where the genes used are derived from mangrove, it is preferable to use higher plants. In producing such transgenic plants, it is more advantageous to insert DNA into vectors to express them in plant cells, to introduce them into plant cells, and to recover the transformation plant cells in order to obtain transgenic plants.

As vectors used in producing transgenic plants, it is preferable to use pBI101 commercialized by Toyobo, or pIG121Hm (Plant J. 6, 271-282, 1994). Although there are no special restrictions on the species of plant cells wherein vectors are introduced, they are considered to be rice plant, wheat, corn, soy, tobacco, carrot. Morphology of plant cells can be protoplast, callus, part of plant organism (leafdisk, hypocotyl) and the like. As a method for introducing vectors into host plant cells, the Agrobacterium method is most preferable, and polyethylene glycole method, electroporation method, particle gun method, and others can also be used (MODEL SHOKUBUTU NO JIKKEN PROTOCOL (Experimental Protocols for plant models), Shujin-sha, 1996).

The ways how plant cells wherein vectors are introduced vary depending on the species of plants. The following processes are carried out when rice plant is taken as an example. The callus is derived from matured seeds, and the obtained callus is infected with Agrobacterium wherein cDNA are introduced. After the period of co-culturing, they are transferred to selective medium to culture. About three weeks later, callus is transferred to redifferentiation medium, and is cultured until redifferntiation. Transformants are recovered by transferring them to pots after 4 or 5 days of acclimatation. (MODEL SHOKUBUTU NO JIKKEN PROTOCOL (Experimental Protocols for plant models), Shujin-sha, 1996). As methods for recovering carrot, tobacco, and others, the methods proposed by Dr. Kato and Dr. Shono are appropriate for each (SHOKUBUTU SOSHIKI BAIYOO NO GIJUTU (Technologies for culturing plant tissues), Asakura-shoten, 1983).

Breeding materials derived from transgenic plants in the present invention can be any one as long as they are derived from the transgenic plants, and more specifically seeds, tuberous roots, ears, mericlone, and the like can be used as materials for culturing and proliferating, depending on the species of plants. Further, it is possible to produce massively transgenic plants in the present invention based on the breeding materials.

In the following, the present invention will be explained with concrete examples. However, it will not be restricted in any way by the concrete examples.

Example 1 Preparation of cDNA Library of Mangrove and Other Halophytes Plants

Suspension culture cell lines of Bruguiera sexangula established by Miura was used as mangrove suspension culture cells (J. Plant Res. 110, 25-29, 1997). The cells are cultured in AA medium containing 100mM of NaCl, separated 120 ml for each flask of 500ml, and performed shaken culture (70 rpm) in a dark room at 26° C. Following the procedure shown below, the cDNA library of mangrove was carried out by using suspension culture cells. First, following the method by Ostrem et al. (Plant Physiol. 84, 1270-1275, 1987), total RNA were extracted, and then poly(A)+RNA was purified

by using Oligotex-dT30<super> (Daiichi Kagaku sha). The purified poly(A)+RNA was used to synthesize cDNA, and cDNA library was constructed by introducing the lambda phage vector λ ZapII (Stratagene). Methods for constructing cDNA library by introducing λ ZapII are well known, and the actual procedure was followed by the manufacturer's manual by Stratagene. As a result, a mangrove cDNA library containing independent clones of 10^6 was successfully constructed. In order to construct cDNA libraries of other halophytes plants such as Avicennia marina (a kind of mangrove), Sueada japonica, Salsola komarovii, Mesembryanthemum crystallinum, leaves of each plant organism are used. The same method was used to construct cDNA library as in the case of mangroves. The obtained cDNA library contained 10^5 to 10^6 of independent clones for each.

Example 2 Determination of the Conditions of Screening cDNA Relevant to Salt Tolerance

The present inventors used the gene expression system of coliforms as a method for screening cDNA relevant to salt tolerance from cDNA library, of mangrove or other halophytes. In other words, the present inventors developed a novel method for acquiring cDNA relevant to salt tolerance by introducing cDNA of mangrove or other halophytes into coliforms and selecting transformed coliforms with their salt tolerance improved. 2YT agar medium containing appropriate concentration of NaCl were used to select transformed coliforms with their salt tolerance improved. Before starting the screening, the present inventors determined the minimal concentration of NaCl inhibiting growth of various coliforms (DH5 α, JM109, HB101, SOLR) in order to select the host coliforms appropriate for the screening. Such coliforms are well-known strains, and they are commercialized by TOYOBO and Stratagene, and others. Although the growth of DH5 α, JM109, and HB101 is remarkably inhibited on the 2YT agar medium containing 1200mM of NaCl; they are able to form colonies. Their growth is completely restricted under 1500mM of NaCl. On the contrary, the growth of SOLR was remarkably inhibited in NaCl of less than 300mM, and completely

inhibited under 400mM of NaCl. These facts show that SOLR is a strain with high sensitivity to salt, and differs from other coliforms in that it does not have a strong mechanism against salt. The fact that salt tolerant mechanisms of the coliforms themselves do not work is very effective in performing the screening. Therefore, the screening of cDNA relevant to salt tolerance from cDNA library was carried out in the following procedure by using SOLR as host coliforms and by fixing the concentration of NaCl at selection agar medium as 400mM.

Example 3 cDNA Screening Relevant to Salt Tolerance From cDNA Library of Mangrove and Other Halophytes

cDNA library of mangrove and other halophytes was introduced into SOLR by inserting pBluescript SK carrying the library with in vivo excision system (Stratagene). The introduction of genes was performed by manufacturer's manual for ZAP-cDNA/Gigapack Cloning Kit (Stratagene). In order to select SOLR introduced with cDNA relevant to salt tolerance from SOLR introduced with cDNA of mangrove and other halophytes, two steps of screening were performed. In the first screening, SOLR introduced with cDNA of mangrove and other halophytes was planted on 2YT agar medium containing 400mM of NaCl, 50 μ g/ml of kanamycin, 50 μ g/ml of ampicilline, and 0.05mM of IPTG, and cultured at 37°C for 20 hours. All colonies obtained under the conditions are inoculated on said agar medium again, and their growth was observed. As a result of this process, 168 clones with salt tolerance improved were obtained on Bruguiera. Almost the same number of clones with their salt tolerance improved were obtained successfully in the transformed coliforms wherein cDNA library of halophytes was introduced. Since there are possibilities of improving salt tolerance of clones derived from host coliforms for some reasons, second screening was performed in the following way.

Plasmids were extracted from each clone obtained by the first screening, and were all reintroduced into SOLRs. Transformants obtained were cultured until log phase on the 2YT liquid medium containing 50 μ g/ml of kanamycin, 50 μ g/ml of ampicillin,

0.05mMof IPTG, diluted series were produced on 2YT liquid medium, and were spotted 25 μl for each on the agar selection medium. After the liquid was blow-dried, they were cultured at 37°C overnight. The result confirmed that 30 clones improved salt tolerance in Bruguiera cDNA library. As a representative, Fig. 1 shows the result of spot experiment of coliforms wherein cDNA shown in Seq. ID No. 1 is introduced. Next, the sequence of bases of cDNA wherein 30 clones were introduced was determined following manufacturer's manual, by using Thermo Sequenase Cycle Sequencing Kit (Amersham) and DNA Sequencer LIC-4000L (LI-COR). The result is that Bruguiera cDNA obtained from 30 clones were classified into 7 classes. More specifically, 23 pieces of cDNA shown in Seq. ID No. 1, one piece of cDNA shown in Seq. ID No. 3, 2 pieces of cDNA shown in Seq. ID No. 5, 2 pieces of cDNA shown in Seq. ID No. 7, one piece of cDNA shown in Seq. ID No. 9, one piece of cDNA shown in Seq. ID No. 11, and one piece of cDNA shown in Seq. ID No. 13 were obtained.

In similar manners, cDNA shown in Seq. ID No. in 15, 17, 19, 35, 63 are obtained from Mesembryanthemum crystallinum, cDNA shown in Seq. ID No.s in 21, 37, 39, 51, 53, 57 are obtained from Suaeda japonic, cDNA shown in Seq. ID No.s as 23, 25, 41, 47, 49, 59, 61 are obtained from Salsola komarovil, and cDNA shown in Seq. ID No.s 27, 29, 31, 33, 43, 45, 55 are obtained from Avicennia marina.

The BLAST Homology Search program was used to search homology of the sequences of amino acids encoding these pieces of DNA. The result is that there are no proteins registered which have homology with the sequence of amino acids shown in Seq. ID No. 2 in the databases such as Swiss protein, PIR, and revealing that the proteins are novel. Therefore, the present inventors named the novel protein encoding the cDNA (total number of amino acids: 141) as mangrin, and the genes as mang1. Next, functional regions of mangrin were determined. As the result of the spot experiment that the subclones were produced by introducing terminal codons into the amino acids of No. 16, 42, 65, 87, 109, 142, and subclones were produced by introducing methionine (and terminal codons were introduced immediately before this) into the amino acids of No. 16, 35, 49, and these obtained subclones were introduced

into SOLRs. The result of the spot experiments of SOLRs shows that the region responsible for salt tolerance is a sequence of amino acids 17 to 86 (Fig. 2).

The sequence of amino acids shown in Seq. ID No. 4 has about 90% of homology with t-complex polypeptide 1 (pir JN0448) from Arabidopsis thaliana. The sequence of amino acids shown in Seq. ID No. 6 has about 80% of homology with Metallothionein-like protein TYPE 2 (EMBL L02306) from Ricinus communis. The sequence of amino acids shown in Seq. ID No. 8 has about 63% of homology with RubB-like DNA helicase (AB024301) from Homo sapiens. The sequence of amino acids shown in Seq. ID No. 10 has about 45% of homology with Ribosomal protein S29 (pir S30298) from Rattus norvegicus. The sequence of amino acids shown in Seq. ID No. 12 has about 90% of homology with Elongation factor eEF-1 alpha chain (pir S66339) from Zea mays. The sequence of amino acids shown in Seq. ID No. 14 has about 70% of homology with cdc21 (pir S26640) from Schizosaccharomyces pombe. Th cDNA shown in Seq. ID No.s 1, 3, 5, 7, 9, 11, or 13 encoding proteins shown in Seq. ID No.s 2, 4, 6, 8, 10, 12, or 14 respectively are considered to have functions of improving salt tolerance in a wide-range group of organisms from procarytes such as coliforms to higher animals because they actually have functions of improving salt tolerance of coliforms.

Likewise, the sequence of amino acids derived from Mesembryanthemum crystallinum shown in Seq. ID No. 16 has 68% of homology with F13O1.15 gene product (gpAC006193_15) from Arabidopsis thaliana. The sequence of amino acids derived from Mesembryanthemum crystallinum shown in Seq. ID No. 18 has 78% homology with H+-transporting ATPase (EC 3.6.1.35) 14K chain (pir T01087) from Arabidopsis thaliana. The sequence of amino acids derived from Mesembryanthemum crystallinum shown in Seq. ID No. 20 has 91% of homology with 40S RIBOSOMAL PROTEIN S20 (pir T12992) from Arabidopsis thaliana. The sequence of amino acids derived from Suaeda japonica shown in Seq. ID No. 22 has 63% of homology with ozone-inducible protein (prf 2316438B) from Atriplex canescens. The sequence of amino acids derived from Salsola komarovil shown in Seq. ID No. 24 has 58% of

homology with GIBBERELLIN-REGULATED PROTEIN 1 PRECURSOR (sp GAS1_ARATH) from Arabidopsis thaliana. The sequence of amino acids derived from Salsola komarovii shown in Seq. ID No. 26 has 99% of homology with ADP-ribosylation factor (gp AF 022389_1) of Vigna unguiculata. The sequence of amino acids derived from Avicennia marina shown in Seq. ID No. 28 has 56% of homology with tuberization-induced protein (prf 2310431A) of Solanum demissum. The sequence of amino acids derived from Avicennia marina shown in Seq. ID No. 30 has 69% of homology with Enod93 protein (gp MSA248334_1) of Medicago sative. The sequence of amino acids derived from Avicennia marina shown in Seq. ID No. 32 has 69% of homology with 40S RIBOSOMAL PROTEIN S21 (pir S38357) of Oryza sative. The sequence of amino acids derived from Avicennia marina shown in Seq. ID No. 34 has 79% of homology with protein phosphatase 2C homolog (AF097667) of Mesembryanthemum crystallinum. It also has 79% of homology with protein phosphotase 2C homolog (AF097667) of Mesembryanthemum crystallinum.

Further, the sequence of amino acids derived from Mesembryanthemum crystallinum shown in Seq. ID No. 36 has 58% of homology with pRIB5 protein (gp RNI7578_1) of Ribes nigrum. The sequence of amino acids derived from Sueada japonica shown in Seq. ID No. 38 has 84% of homology with tonoplast intrinsic protein (pir T12439) of Mesembryanthemum crystallinum. The sequence of amino acids derived from Sueada japonica shown in Seq. ID No. 40 has 86% of homology with phosphoethanolamine N-methyltransferase (gp AF237633_1) of Spinacia oleracea. The sequence of amino acids derived from Salsola komarvii shown in Seq. ID No. 42 has 83 % of homology with phosphoenolpyruvate carboxylase (gpu SWI17843_1) of Selenicereus wittii. The sequence of amino acids derived from Avicennia marina shown in Seq. ID No. 44 has 84% of homology with putative chaperonin (gp ATAC021640_16) of Arabidopsis thaliana. The sequence of amino acids derived from Avicennia marina shown in Seq. ID No. 46 has 88% of homology with hypothetical protein T5F17.40 (pir T10653) of Arabidpsis theliana.

Further, the sequence of amino acids derived from Salsola komarvii shown in Seq.

ID No. 48 has 63% of homology with cysteine proteinase inhibitor (pir T07139) of Glycine max. The sequence of amino acids derived from Salsola komarvii shown in Seq. ID No. 50 has 87% of homology with nucleotide sugar epimerase-like protein (gp ATCHRIV73_17) of Arabidopsis thaliana. The sequence of amino acids derived from Sueada japonica shown in Seq. ID No. 52 has 57% of homology with putative protein (gp ATT20K12_12) of Arabidopsis theliana. The sequence of amino acids derived from Sueada japonica shown in Seq. ID No. 54 has 78 % of homology with putative WD-40 repeat protein (gp AC006569_14) of Arabidopsis thaliana. The sequence of amino acids derived from Avicennia marina shown in Seq. ID No. 56 has 75 % of homology with cdc2MsE gene product (gp MSCDC2MSE_1) of Medicago sativa. The sequence of amino acids derived from Sueada japonica shown in Seq. ID No. 58 has 39% of homology with putative protein (gp ATF17C15_9) of Arabidopsis thaliana. The sequence of amino acids derived from Salsola komarovii shown in Seq. ID No. 60 has 66% of homology with transcription factor E2F (prf 2601241A) of Nicotiana tabacum. The sequence of amino acids derived from Salsola komarovii shown in Seq. ID No. 62 has 34% of homology with hypothetical protein T26B15.5 (pir T02548) of Arabidopsis thaliana. The sequence of amino acids derived from Mesembryanthemum crystallinum shown in Seq. ID No. 64 has 30% of homology with Homo sapiens cDNA FLJ10298 fis, clone NT2RM1001115, weakly similar to ENDOCHITINASE 2 PRECURSOR (EC 3.2.1.14) (pir T02548).

Example 4 Effects of Mangrove cDNA in Yeast

The pBluescript SK cloned with cDNA shown in Seq. ID No. 1 was digested by restriction enzymes EcoRI or NotI, and was subjected to agarose gel electrophoresis. About 1kb fragment obtained here was excised, and was purified with GENECLEAN kit (BIO101). By using Ligation Kit ver2 (TAKARA), the fragments were introduced into yeast expression vectors pYES2 (Invitrogen) which were digested with restriction enzymes EcoRI and NotI. Next, the vectors are introduced into yeast by means of electoroporation. Saccharomyces cereviciae YM4271 (Clontech) was used as yeast.

SD agar medium which does not contain uracil (hereafter, -UraSD agar medium) was used to select transformed yeast. The salt tolerance of transformed yeast was evaluated in the following way. Transformed yeast was cultured on –UraSD medium until the late period of the logarithmic growth phase inoculated on –UraSD medium containing 1200mM of NaCl and –Ura SD medium not containing NaCl (the primary concentration is OD600=0.1), and was subject to shaking culture at 30°C. The cell suspension was extracted every 24 hours, and its absorbance was measured. UV-1200 (SHIMAZU SEISAKUSHO) was used to measure the absorbance. The same measurement was carried out to make comparison with yeast wherein only pYES2 vectors were introduced. Fig. 3 shows the result of the measurements. As obvious from Fig.3, the cDNA in yeast obtained by the screening showed similar function of salt tolerance as the ones in coliform.

Example 5 Effects of Mangrove cDNA in Tobacco Cultured Cells

The pBluescript SK cloned with cDNA shown in Seq. ID No. 1 was digested by the restriction enzymes Xba1 and Xho1, and was subjected to agarose gel electrophoresis. About 1kb fragment obtained here was excised, and purified by GENECLEAN kit (BIO101). By using Ligation Kit ver2 (TAKARA), the fragments were introduced into the restriction enzymes EcoRI and NotI sites of plant expressing vector pBI101 (EMBOJ 6, 3901-3907, 1987). The obtained plasmids were introduced into Agrobacterium tumefaciens, by the electoroporation method, and were infected to tobacco cultured cells (Nicotiana tabacumL. Cv. Bright Yellow 2). It is well known to use Agrobacterium tumefaciens in introducing genes into plant cells. Here, Agrobacterium tumefaciens EHA 101 was used as Agrobacterium, and the method of An was used (Plant Physiol. 79, 568-570, 1985). The salt tolerance of transformed tobacco cultured cells were evaluated in the following way. Callus of transformed tobacco cultured cells were collected, and was cultured on Lins-Mayer medium until the late period of the logarithmic growth phase. The obtained Callus were separated 1ml for each on 45 ml of Lins-Mayer media which were prepared to be 0, 100, or 150mM of

NaCl concentrations, and were subjected to shaking culture at 26°C. On days 7 to 13 after the culture began, cells were collected from cell suspension, and their wet weight was measured. Tobacco culture cells wherein pBI101 was used instead of mangrove cDNA for introduction of GUS genes were measured in the same manner. Fig. 4 shows the result of the measurements. As is obvious from Fig.4, the cDNA in tobacco culture cells obtained by the screening showed similar function of salt tolerance as the ones in coliform.

Example 6 Effects of Mangrove cDNA in Tobacco (Plants)

Plasmids obtained by Example 5 were introduced into Agrobacterium tumefaciens by electoroporation, and the plasmids were infected to tobacco leafdisks. It is well known to use Agrobacterium tumefaciens in introducing genes into tobacco leafdisks. Here, Agrobacterium tumefaciens EHA 101 was used as Agrobacterium, and it was carried out following the method described in Shokubutu Saiboo Koogaku Nyuumon (Introduction to plant cell engineering) (Japan Scientific Societies Press, 1998). The salt tolerance of transformed tobacco (plant organism) was evaluated in the following way. Transformed tobacco was planted on MS agar medium where the concentration of NaCl was prepared to be 150 mM, and was cultured at 26°C under lighting of the lighting cycle (light: 16 hours/ dark: 8 hours). The growth of the plant organisms after 30 days of culture was observed, and its salt tolerance was evaluated. Tobacco culture cells into which GUS genes were introduced by pBI101 rather than mangrove cDNA were examined in the same fashion. Fig. 5 shows the result (see also the photos). As obvious from Fig. 5, the roots, leaves, and stems of the tobacco plant organisms obtained from the result of screening show a high rate of growth. This confirms that cDNA obtained from the result of screening have functions of improving salt tolerance at the level of plant organism.

Example 7 Effects of Mangrove cDNA Against Various Environmental Stresses

(1) Thermal Stress

SOLR into which cDNA was cloned shown in Seq. ID No. 1 and pBluescript SK was introduced was cultured in 2YT liquid medium containing 50 μ g/ml of kanamycin, 50 μ g/ml of ampicillin, 0.05mM of IPTG at 37°C, and 40°C. As a control, SOLR wherein pBluescript SK, a vector, was introduced as a vector was examined in the same manner. Fig. 6 shows the result. As obvious from Fig. 6, cDNA obtained from the result of screening have functions of improving thermal tolerance.

(2) Osmotic Pressure Stress

SOLR wherein cDNA was cloned shown in Seq. ID No. 1 and pBluescript SK was introduced was cultured in 2YT liquid medium containing 50 μ g/ml of kanamycin, 50 μ g/ml of ampicillin, 0.05mM of IPTG until the logarithmic growth phase. Dilution series were produced on 2YT liquid medium, and were spotted each for 25 μ l on 800mM of 2YT agar medium. After the liquid was air-dried, culture was carried out at 37°C overnight. Fig.7 shows the result. As seen from Fig. 7, it was confirmed that cDNA obtained from the screening have functions of improving osmotic pressure tolerance.

(3) Freezing Stress

SOLR wherein cDNA was cloned shown in Seq. ID No. 1 and pBluescript SK was introduced was cultured in 2YT liquid medium containing 50 μ g/ml of kanamycin, 50 μ g/ml of ampicillin, 0.05mM of IPTG until the logarithmic growth phase, and was diluted to be 5000 cells/25 μ l on 2YT liquid medium. The cells were transferred to plastic tubes, and they were frozen by liquid nitrogen for 3 minutes and melt at 37° C for 10 minutes repeatedly. Part of the cell body (25 μ l) was taken when melted, and SOLR was spotted on 2YT agar medium containing 50 μ g/ml of kanamycin, 50 μ g/ml of ampicillin, 0.05mM of IPTG. As a control, SOLR wherein pBluescript SK, a vector, was introduced as a vector was examined in the same manner. Fig. 8 shows the result. As obvious from Fig. 8, cDNA obtained from the screening have functions of improving tolerance against freezing.

Example 8. Molecular Evolution of Proteins Having the Activity of

Improving Environmental Stress Tolerance

It was attempted to introduce random mutants into the region considered to be mangrin minimal functional region (mangrin core) by performing PCR using plasmids cloned with regions considered to be mangrin cDNA minimal functional region (Amino Acid Number: 17-86) as a template, as shown in Fig. 2. As primers, 5'-GCTCTGAGAACCGTCTAGACTTAGATGAAGGTG-3' shown in Seq. ID No. 65, and 5'-TCTCTCGTTCATCTCGAGCTATTACAGCTC-3' shown in Seq. ID No. 66 were used. These primers were designed to amplify mangrin core in a way that initiation codon and termination codon, and restriction enzymes (Xba1, Xhol) sites on the outer sides of the codons, were added. In performing PCR, TAKARA Taq (Mg^{2+} free buffer) (TAKARA) was used as a DNA polymerase. The PCR reactive solution was added to the accompanying buffers to be in the ratios of 1.0mM of $MgCl_2$, 0.5mM of $MnCl_2$, 0.25mM of dNPT Mixture, where 2pmol/10μl of each primer and template DNA to 10pg/10μl are added. The condition of reaction temperature was set as 92°C for 30 seconds, 50°C for 30 seconds, and 72°C for 90 seconds. This cycle was repeated 30 times, and DNA fragments wherein random mutants were introduced into margin core were obtained. The DNA fragments obtained were digested with Xbal and Xhol, and these are cloned to the vectors (pBluescript SK) already digested with Xbal and Xhol. These were introduced into SOLR, and were selected with the growth on agar medium containing 450mM of NaCl as an index. Although the sequence of bases are mutated, mutant mangrin core maintaining or improving activity of improving salt tolerance was selected. The sequence of bases is determined on part of obtained clones, and the activity of improving salt tolerance was evaluated by spot test. As shown in Fig. 9, the result shows that 2 clones (c-52, c-80) of a sequence of bases are mutated. Among them, c-80 is mutated in the sequence of amino acids, and this is considered to be a factor of improving functions of salt tolerance in mangrin core. Further, as seen from Fig. 10 for comparing functions of improving stress tolerance, it can be used to improve functions of environmental stress tolerance genes.

INDUSTRIAL APPLICABILITY

The present invention will be an effective means to improve tolerance against environmental stress for various animals. Specifically, the plants whose tolerance against environmental stress tolerance is improved can grow in salt damaged lands, cold regions, deserts, and oceans, where they are difficult to grow. From this fact, it is expected that the amount of agricultural products will increase due to the expansion of farmland. Further, the plants whose environmental stress tolerance is improved contribute to the suppression of global warming due to the global increase of CO_2 level globally, as well as to the increase of greenery areas and greening of deserts.